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Saliva Testing for HPV in Oropharyngeal Squamous Cell Carcinoma (OPSCC): A Diagnostic Accuracy Study

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Key Words: HPV, p16 immunohistochemistry, oropharyngeal squamous cell carcinoma, DNA in-situ hybridisation, saliva testing

Abstract

Background

New cases of oropharyngeal squamous cell carcinoma (OPSCC) are routinely tested for HPV. HPV in saliva can be detected with PCR, but its clinical applicability in the context of OPSCC remains unknown.

Methods

Forty-six consecutive patients diagnosed with OPSCC had pre-treatment saliva specimens collected. PCR for HPV on saliva was compared to p16 IHC and HPV DNA in-situ hybridisation (ISH) on surgical biopsies.

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Results

The sensitivity and specificity of saliva testing when compared to the reference test of p16 IHC and HPV DNA ISH was 72.2% and 90% and positive and negative predictive values were 96.3% and 47.4%. There were no adverse events. Time from last meal, smoking, alcohol drinking and physical exercise did not impact on results.

Conclusions

Saliva testing is a promising test to detect HPV in patients with OPSCC. A positive result could avoid the need for surgical biopsies, thereby reducing costs, patient morbidity, and expedite treatment.

Introduction:

Oropharyngeal squamous cell carcinoma (OPSCC) which affects the tonsils and tongue base is traditionally associated with excess alcohol consumption and smoking. Despite a reduction in smoking and alcohol consumption, the UK incidence of OPSCC doubled between 1990 and 2006 and then again between 2006 and 2010[1]. This increase is mirrored in many developed countries[2], with approximately 63,000 new cases of OPSCC reported annually [3]. The majority are now attributed to HPV infection (HPV+ve)[2]. A worldwide meta-analysis has shown that HPV associated OPSCC has increased from 40.5% before 2000 to 72.2% after 2005 [4]. HPV+ve OPSCC is set to overtake the incidence of HPV associated cervical cancer by 2020 [5] and is a major health epidemic in the western world [6].

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HPV detection is emerging as a biomarker for patients with OPSCC. The presence of HPV in OPSCC can predict prognosis and determine suitability for entry into de-escalation clinical trials aimed at improving long-term quality of life[7,8]. In fact, HPV+ve and HPV-ve OPSCC are staged separately in the latest edition of the AJCC cancer staging manual[9]. Whilst current treatments are not based on HPV status (outside of clinical trials), the new staging system is likely to have an impact.

HPV testing using p16 immunohistochemistry (IHC) on surgical or core biopsies is considered the standard of care for patients with OPSCC, and is recommended in all patients by the UK National Institute for Health and Care Excellence (NICE)[10]. p16 IHC is also the standard of care utilised by the AJCC in determining the presence of HPV in patients with OPSCC[9]. Other techniques for HPV testing including the sensitivity and specificity of these tests are described in Table 1.

HPV testing for OPSCC is typically performed on surgically obtained biopsies performed under general anaesthesia or on core biopsies of suspected lymph node metastasis obtained under ultrasound guidance. These investigations have associated costs and risks including pain, bleeding, infection, dental injury, oesophageal perforation and rarely airway compromise. The procedures can result in delays to the patient pathway particularly when a diagnosis of squamous cell carcinoma is first made with fine needle aspiration cytology (FNAC). In these cases a supplementary surgical or core biopsy is needed from the primary site or nodal metastasis to confirm p16 status. With existing pressures on cancer diagnostic services[11], the process from initial presentation to establishing the HPV status on a biopsy of a patient with OPSCC may take weeks.

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HPV is detectable in the saliva of patients with OPSCC using PCR. This non-invasive, relatively inexpensive test, without side effects and taken at the point of first patient contact could allow for the HPV status of patients to be established much earlier. Salivary HPV testing has the potential to mitigate the need for invasive biopsies performed solely for the purpose of HPV detection, and could form a routine part of screening and risk stratification in patients suspected of OPSCC. To our knowledge, there is no prospective diagnostic accuracy study directly comparing the efficacy of oral rinse testing to the clinically accepted standards of p16 IHC and DNA ISH in patients with OPSCC.

Aims:

The aim of this study was to determine the sensitivity and specificity of oral rinse (OR) testing for HPV in patients with OPSCC.

Materials and Methods:

A prospective diagnostic accuracy study estimating the sensitivity and specificity of oral rinse testing using PCR for HPV to p16 IHC and DNA ISH in patients with OPSCC was conducted. The study flow diagram is shown in Figure 1. Both QUADAS-2 [12] and STARD criteria [13] were adopted. Ethical approval was sought in collaboration with the Oxford Radcliffe Biobank (Ref No. 09/H0606/5+5).

Between September 2015 and June 2016, sixty-three patients were referred to the Oxford University Hospitals (OUH) Head and Neck Cancer Service with suspected OPSCC. Consecutive adult patients (aged $\geq$18) with mental capacity and suspected OPSCC were invited to

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participate. All patients met initial inclusion criteria, one patient declined consent. Oral rinse (OR) specimens and demographic data were collected from the 62 consenting patients prior to commencing diagnostic assessment and cancer treatment. 47 patients were subsequently confirmed to have a diagnosis of OPSCC and were included in this study.

A standard operating procedure was developed whereby patients were asked to 'gargle' 10mls of sterile sodium chloride solution for 20-30 seconds. This was collected in a sterile universal container and stored within 2 hours of collection at -80°C. OPSCC tumour biopsies were performed under general anaesthesia and formalin fixed, paraffin embedded tissue was assessed for routine diagnostic histopathology. Immunostaining for p16 was performed on all cases and confirmatory HPV DNA ISH on twenty-nine. HPV DNA ISH was performed when felt to be of clinical value, this was at the discretion of the reporting Consultant Head and Neck pathologist.

p16 IHC was considered positive when >70% of cells showed strong nuclear and/or cytoplasmic staining [10]. HPV DNA ISH was carried out using the Ventana INFORM HPV III Family 16 Probe (B) (MDCI Ltd, West Sussex UK). The probe cocktail has demonstrated affinity to the following genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66.

The index test *(Linear Array HPV Genotyping Test Kit – Roche ©)* was performed by the OUH Molecular Diagnostics Department in accordance with the manufacturers instructions on all 47 samples. HPV DNA was extracted from each OR sample by lysing cells in denaturing conditions at elevated temperatures. PCR amplification for 37 different HPV subtypes was performed using the provided 'master mix'. Hybridization

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was used to label oligonucleotide probes before using a Streptavidin-Horseradish peroxidase conjugate to identify HPV status and sub-type using the Linear Array HPV Genotyping Test Reference Guide. Suitable controls were used at each stage of the process. There was one OR test failure meaning that 46 of 47 eligible patients were included in the final analysis. Both reference and index tests were conducted by qualified technicians blinded to the clinical presentation and results.

When the results of p16 IHC and DNA ISH were combined to determine HPV status a sample was considered positive if it was either p16 IHC positive with no HPV DNA ISH test, p16 IHC positive and HPV DNA ISH positive or p16 IHC negative and DNA ISH positive. A sample was considered HPV negative if it was p16 IHC negative with no HPV DNA ISH test, p16 IHC negative and HPV DNA ISH negative or p16 IHC positive and DNA ISH negative.

Statistical Analysis

A power calculation was conducted using Stata 13 (StataCorp LP) software to determine a sample size of 45 for 80% power to determine results with a standard error of 5% and 10% confidence intervals. All statistical analyses were pre-determined and performed using Prism 6 for Mac OS X (© 1994-2015 GraphPad Software, Inc). The demographic differences in the patient population based on p16 IHC and 'true vs false' OR results were analysed using a Fisher's exact test when categorical values were less than 5 and a Chi^2 test when they were greater than 5 or when there were more than 2 categories. A result was considered significant when p was ≤ 0.05. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) and their 95% confidence intervals were calculated.

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Results

The mean age of participants was 58.8 years (range; 37-80) (see Table 2). There were 35 males (76.1%) and 11 females (23.9%); all patients were of Caucasian descent. The mean alcohol consumption was 19.8 units per week; there were 34 (73.9%) current or ex tobacco smokers with an average 23.2 pack year (range; 1-50) smoking history. The majority of patients presented with T1/2 tumours (71.7%) with advanced local metastases in 78.3% (N2/3). One patient (2.2%) had distant metastases. Five patients had Stage 1, 28 Stage 2 and 13 Stage 3 disease (ICON-S).

There were 34 (73.9%) p16 positive (+ve) and 12 (26.1%) p16 negative (-ve) samples. There were no statistically significant differences between the two groups in terms of age, sex, co-morbidities, alcohol consumption, smoking status, tumour size or distant metastases. Patients with p16+ve OPSCC presented with a higher N stage than those that were p16-ve (85.3% vs 58.3% p=0.05).

Disease Prevalence, Sensitivities, Specificities and Predictive Values for HPV

The patient specimens were categorised into 3 groups for analysis: those that had p16 IHC and OR testing (n=46), those with DNA ISH and OR testing (n=29) and those that had p16 IHC with or without DNA ISH and OR testing (n=46). The patients and test results for each group are summarised in Figure 2 and Table 3.

Using the previously described method for combined p16 IHC and DNA ISH as the reference test there were 36 HPV positive (78.3%) and 10 HPV negative (21.7%) samples. Of the 36 HPV positive samples 26 were positive and 10 negative on OR testing. Out of the 10 HPV negative

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samples 1 was positive and 9 negative on OR testing. The prevalence of HPV was 78.3% (63.6%-89.1% 95% CI). The sensitivity and specificity of OR testing when compared to the reference test of p16 IHC with and without HPV DNA ISH was 72.2% (54.8%-85.8% 95% CI) and 90% (55.5%-99.8% 95% CI) and PPV and NPV were 96.3% (81.0%-99.9% 95% CI) and 47.4% (24.5%-71.1% 95% CI).

Identification of HPV Sub-types

Oral rinse testing using the Linear Array HPV Genotyping Test Kit (Roche ©) allows identification of up to 37 different HPV subtypes. Twenty-seven out of 46 'oral rinse' tests were positive for HPV (see Figure 3). There was 1 false positive result that suggested the presence of HPV 16 (p16 IHC and HPV DNA ISH negative). HPV 16 alone was detected in 21 samples (77.8%); HPV 18 alone in 1 sample (3.7%), whilst the remaining 5 samples (18.5%) all contained HPV 16 alongside other HPV subtypes. The combinations detected were HPV 16/33/35/52/58, HPV 16/31/35, HPV 16/53, HPV 16/84 and HPV 16/55/73/84.

Evaluating Potential Confounding Factors

To evaluate potential patient demographic, tumour or environmental factors that may have resulted in a false test result additional patient specific information was collected including time of last alcoholic drink, smoke, meal and exercise (see Table 4). Using the reference standard of combined p16 IHC and HPV DNA ISH as previously described there were 35 patients (76.1%) with a true positive or negative result and 11 (23.9%) with a false positive or negative result. There was no statistically significant difference between the two groups in terms of age, co-morbidities, T/N/M stage, last meal/smoke/alcohol or exercise.

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Adequacy of Samples and Adverse Events

One (2.1%) of 47 oral rinse tests failed to provide a result despite confirming adequacy of DNA and repetition; the cause for this remains unclear. Whilst some patients reported that the taste of sodium chloride was unpleasant and some needed to repeat the test due to spillage, there were no adverse events associated with oral rinse testing.

Discussion

Our study has demonstrated that OR testing using PCR to detect HPV in patients with OPSCC has sensitivity, specificity, positive and negative predictive values of 72.2%, 90%, 96.3% and 47.4% respectively. Whilst the NPV is low making interpreting a negative result unreliable, with one sample failure and no adverse events reported this study demonstrates that PCR on oral rinse specimens is a viable method for determining HPV in OPSCC. Smoking, alcohol consumption, last meal and exercise do not appear to affect the accuracy of OR testing meaning that specific pre-test preparation is not required.

Testing for HPV in OPSCC is increasingly important as HPV status can provide important diagnostic and prognostic information. Testing needs to be sensitive and specific, reproducible and validated. There is considerable debate as to the optimum test for HPV[14]. The commonly described techniques of p16 IHC, HPV DNA ISH and PCR on tumour tissue have associated advantages and disadvantages[15]. HPV DNA ISH is able to demonstrate HPV integration within tumour cells, but as it detects DNA and not mRNA it also does not confirm transcriptional activity[15]. p16 IHC, although not 100% specific is emerging as a prognostic marker for OPSCC in its own right [16,17]. It is considered a surrogate marker of HPV transcriptional activity[15]. There are associated limitations, Wasylyk et al (2013) [18] reported p16 IHC alone confirmed a significant number of false positives for HPV when compared to p16 IHC supplemented by

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either PCR or DNA/RNA ISH. PCR for HPV is highly sensitive; whilst this has obvious advantages it is not always possible to tell if the DNA detected relates to the tumour cells or surrounding normal tissue[15].

OR testing offers an alternative to conventional HPV testing in OPSCC with potential benefits of reduced patient morbidity, shorter time to diagnosis and reduced cost. It is also limited by the highly sensitive nature of PCR, whereby detected HPV DNA might not relate to tumour tissue, and instead represent a bystander infection.

The sensitivity of salivary testing however has been questioned with some authors reporting an inability to detect HPV from OR specimens in patients with known HPV+ve OPSCC [19]. Zhao et al [20] detected HPV in 50% (21 of 42 specimens) of salivary samples from patients with HPV+ve OPSCC using PCR. Whilst Wang et al[21] found that saliva was inferior to plasma when testing for HPV in patients with OPSCC. They detected HPV in 40% of their salivary specimens and 86% of their plasma specimens. Our results contradict these findings; whilst plasma testing for HPV was not performed we were able to detect HPV in the saliva of HPV+ve cases in 72.2% of cases. This could relate to the way samples were collected, processed and stored in the studies.

Although the results of our study, which to our knowledge is the first of its kind, are promising, it is important to consider the results of PCR testing for HPV using other non-invasive biopsies. Channir et al [22] recently utilised PCR on fine needle aspirates (FNAs) of patients with OPSCC reporting 94.7% sensitivity and 100% specificity whilst Brogile et al [23] used PCR on oropharyngeal brush cytology specimens from patients with OPSCC reporting sensitivity and

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specificity values of 83% and 94%. Although these results seem superior to OR testing, FNA assessment and brush cytology analysis have limitations. Specifically FNAs are limited by the quality of the sample obtained which may be operator dependant [24] whilst inadequate sampling from oropharyngeal brush cytology is high (45.7%) [25]. Both tests require further clinical validation.

Unlike other diagnostic accuracy studies utilising non-invasive biopsies to determine HPV status in OPSCC this study was carried out prospectively using QUADAS-2 and STARD criteria to limit bias and mirror clinical practise. Patients were included consecutively, analyses predetermined and participants closely reflected those seen in clinical practise e.g. HPV prevalence >70%, no involuntary exclusions. Both reference and OR tests were performed by qualified technicians blinded to the study objectives and other test results to limit bias.

This study was limited by the lack of E6/7mRNA testing considered the research 'gold standard' for HPV detection in OPSCC [26]. Whilst the clinical standards of p16 IHC and DNA ISH were utilised as reference tests all included samples did not have HPV DNA ISH performed. This could have introduced bias in the results and underestimated the specificity and negative predictive value of OR testing. Nonetheless, the reference test utilised represented clinically acceptable standards for HPV detection in OPSCC. Separate analyses were performed for p16 IHC alone and p16 IHC supplemented by DNA ISH when clinically indicated.

Given the negative impact of delayed diagnosis and treatment, OR testing could allow for immediate HPV testing at the primary clinical presentation even before specialist consultation. The relatively high specificity and PPV and low NPV mean that a positive OR test could be relied upon whilst a negative test would require additional conventional testing. General practitioners

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could take samples from patients at the time of specialist referral so that HPV status could be available at the first specialist assessment. Patients presenting with an oropharyngeal lesion and neck nodes (as in most cases of OPSCC) could then have a core biopsy performed at the specialist consultation followed by cross-sectional imaging for staging. These results could provide the treating clinician with a tissue diagnosis, HPV status (if OR positive) and radiological stage. In selected cases, for example when a patient is deemed medically unfit for surgery, this could be sufficient to plan treatment and avoid the risks and time associated with surgical biopsies.

There is a need for large prospective studies that look not only at the diagnostic accuracy of HPV detection in OPSCC but also at recurrence and the role of HPV testing in surveillance. It may be that OR testing alone may not be sufficient and require combination with other non-invasive tests. Ahn et al[27] recently tested both saliva and serum to detect HPV in recurrent OPSCC with >90% specificity and 69.5% sensitivity. Future studies should compare all non-invasive diagnostic materials for HPV including brush cytology, FNA and serum plasma to both clinical and research standards for HPV detection e.g. DNA ISH, RNA ISH, p16IHC and HPV DNA PCR.

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Table1: Sensitivity and specificity of clinically available tests for HPV in OPSCC (Schache et al 2011) [13]**.**

Test	Sensitivity	Specificity
P16 IHC	94-100%	79-82%
PCR	97%	87%
DNA ISH	89%	89%

Table 2: Patient Demographics and Tumour Characteristics by p16 Status

		Tumour HPV Status, No.(%)		
	Total Patients, No.(%) N=46	p16 +ve,No.(%) (n=34)	p16 -ve, No.(%)(n=12)	P- Value
Sex				
Male	35 (76.1)	25 (73.5)	10 (83.3)	0.70
Female	11(23.9)	9 (26.5)	2 (16.7)	
Age, (years)				
>60	18 (39.1)	12 (35.3)	6 (50)	0.37
≤60	28 (60.9)	22 (64.7)	6 (50)	
ASA grade				
1/2	42(91.3)	32(94.1)	10(83.3)	0.28

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3/4/5	4 (8.7)	2(5.9)	2(16.7)	
Ethnicity				
Caucasian	46 (100)	34 (100)	12 (100)	n/a
Drinking Status				
Nil	6 (13)	5 (14.7)	1 (8.3)	0.12
1-21 units/week	29 (63)	22 (64.7)	7 (58.3)	
21-40 units/week	7 (15.2)	6 (17.6)	1 (8.3)	
>40 units/week	4 (8.7)	1 (2.9)	3 (25)	
Smoking status				
Never	12 (26.1)	10 (29.4)	2 (16.7)	0.44
Ex-smoker	21 (45.7)	16 (47.1)	5 (41.7)	
Current	13 (28.3)	8 (23.5)	5 (41.7)	
T Classification				
T1/2	33 (71.7)	25 (73.5)	8 (66.7)	0.71
T3/4	13 (28.3)	9 (26.5)	4 (33.3)	
N Classification				
N0/1	10 (21.7)	5 (14.7)	5 (41.7)	0.05
N2/3	36 (78.3)	29 (85.3)	7 (58.3)	
M classification				
M0	45 (97.8)	33 (97.1)	12 (100)	1.00
M1	1 (2.2)	1 (2.9)	0 (0)	

Abbreviations: n/a, not applicable; ASA, American Society of Anaesthesiologists Performance Status.

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Table 3: Sensitivity, Specificity, Disease Prevalence and Predictive Values of 'oral rinse testing' against clinical standard tests of (A) p16 IHC (B) HPV DNA ISH and (C) Combined p16 IHC/DNA ISH

		HPV Prevalence (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
A	'oral rinse' vs p16 IHC, % (95% CI)	73.9(58.9-85.7)	73.5(55.6-87.1)	83.3(51.6-97.9)	92.6(75.7-99.1)	52.6(28.9-75.6)
B	'oral rinse' vs HPV DNA ISH, % (95% CI)	72.4(52.8-87.3)	66.7(43-85.4)	87.5(47.4-99.7)	93.3(68.1-99.8)	50.0(23.0-77.0)
C	'oral rinse' vs p16 IHC +/- HPV DNA ISH, % (95% CI)	78.3(63.6-89.1)	72.2(54.8-85.8)	90(55.5-99.8)	96.3(81.0-99.9)	47.4(24.5-71.1)

Abbreviations: CI, confidence interval; HPV, human papilloma virus; DNA ISH, DNA in situ hybridisation; p16 IHC, p16 immunohistochemistry; PPV, positive predictive value; NPV, negative predictive value.

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Table 4: Comparing patient differences between 'True' and 'False' results

		Oral Rinse Test Result		
	Total Patients, No.(%) N=46	True +ve/-ve, No.(%) (n=35)	False +ve/-ve, No.(%) (n=11)	P- Value
Age, (years)				
>60	18(39.1)	16(45.7)	2(18.2)	0.16
≤60	28(60.9)	19(54.3)	9(81.8)	
ASA				
1/2	42(91.3)	31(88.6)	11(100)	0.56
3/4/5	4(8.7)	4(11.4)	0(0)	
T Classification				
T1/2	33(71.7)	24(68.6)	9(81.8)	0.47
T3/4	13(28.3)	11(31.4)	2(18.2)	
N Classification				
N0/1	10(21.7)	8(22.9)	2(18.2)	0.99
N2/3	36(78.3)	27(77.1)	9(81.8)	
M classification				
M0	45(97.8)	34(97.1)	11(100)	0.99
M1	1(2.2)	1(2.9)	0(0)	
Last Alcoholic Drink				
Never	16(34.8)	12(34.3)	4(36.4)	0.82
< 6 hours	1(2.2)	1(2.9)	0(0)	
6-24 hours	20(43.5)	16(45.7)	4(36.4)	
> 24 hours	9(19.6)	6(17.1)	3(27.3)	
Last Smoke				
Never	34(73.9)	26(74.3)	8(72.7)	0.40

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< 6 hours	7(15.2)	4(11.4)	3(27.3)	
6-24 hours	2(4.3)	2(5.7)	0(0)	
> 24 hours	3(6.5)	3(8.6)	0(0)	
Last Meal				
< 2 hours	7(15.2)	4(11.4)	3(27.3)	0.30
2-6 hours	25(54.3)	21(60)	4(36.4)	
> 6 hours	14(30.4)	10(28.6)	4(36.4)	
Last Exercise				
< 6 hours	4(8.7)	4(11.4)	0(0)	0.44
6-24 hours	10(21.7)	8(22.9)	2(18.2)	
> 24 hours	32(69.6)	23(65.7)	9(81.8)	

Abbreviations: n/a, not applicable; ASA, American Society of Anaesthesiologists Performance Status.

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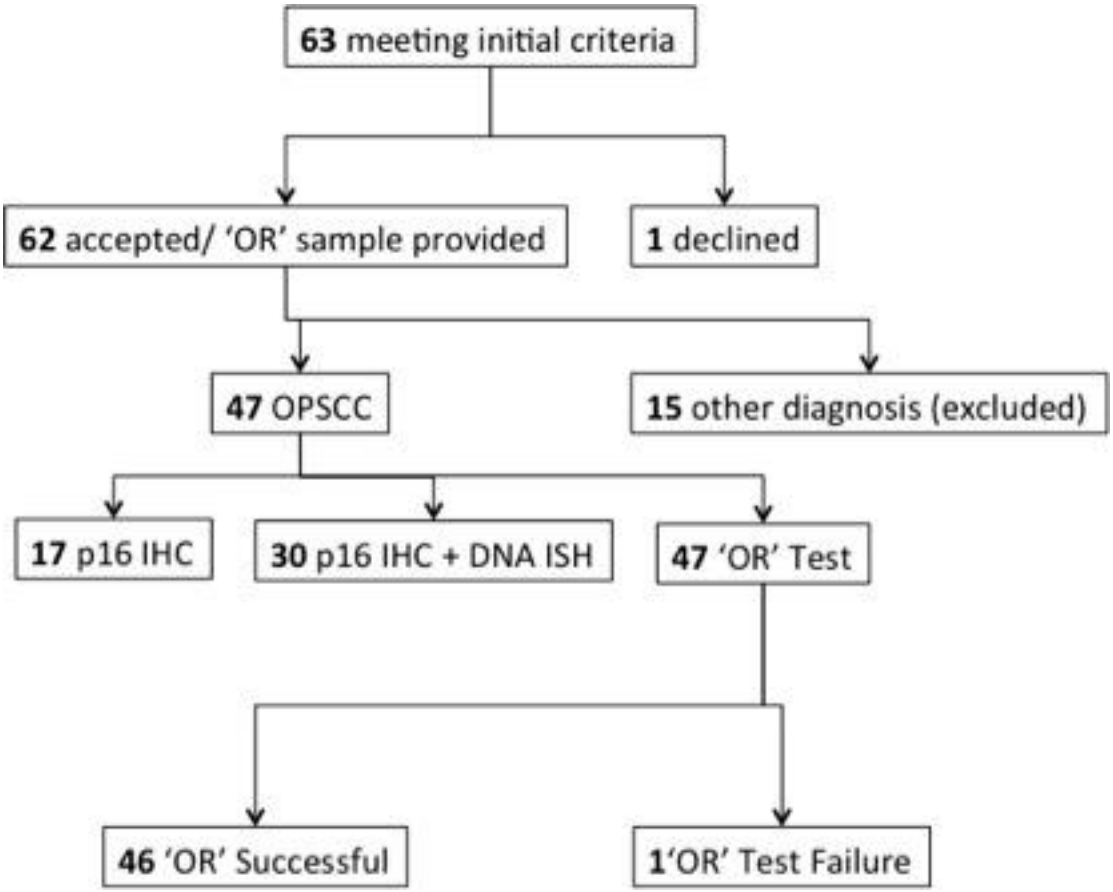

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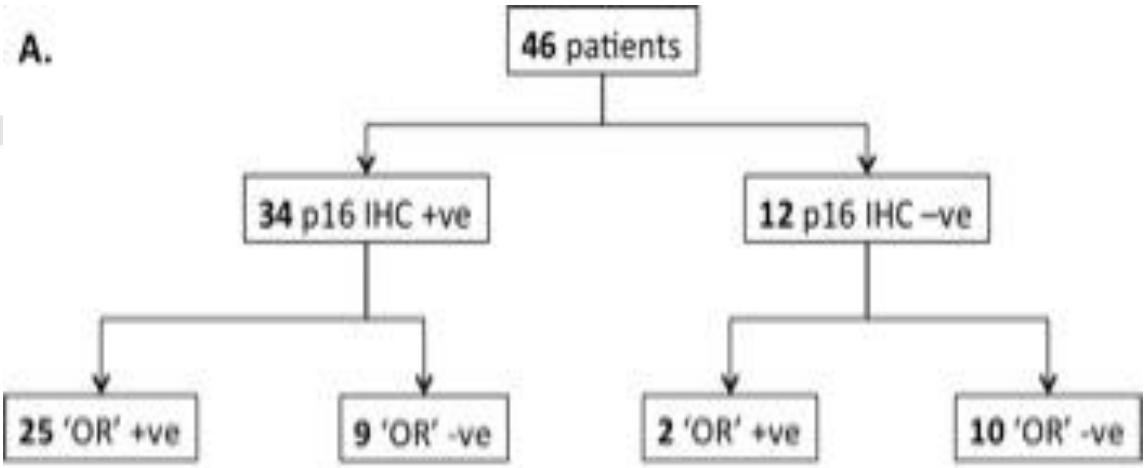

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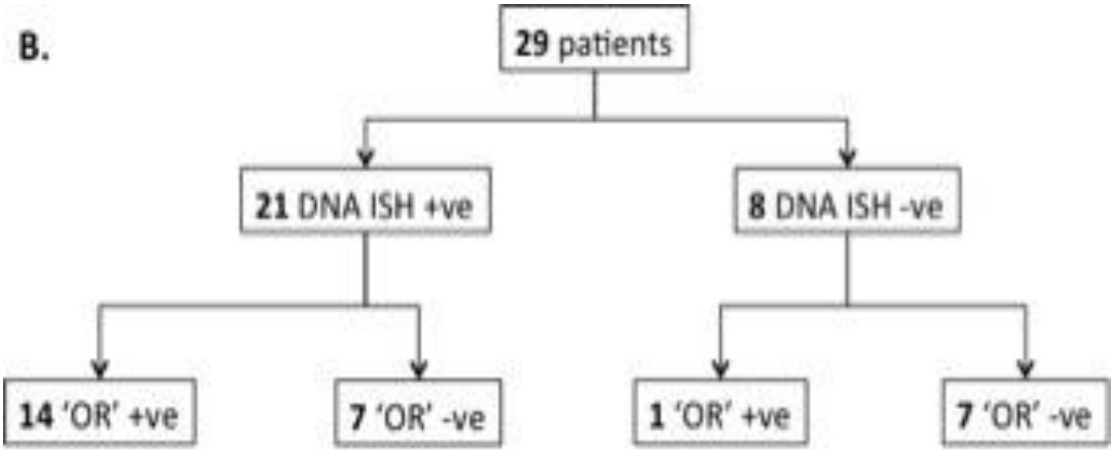

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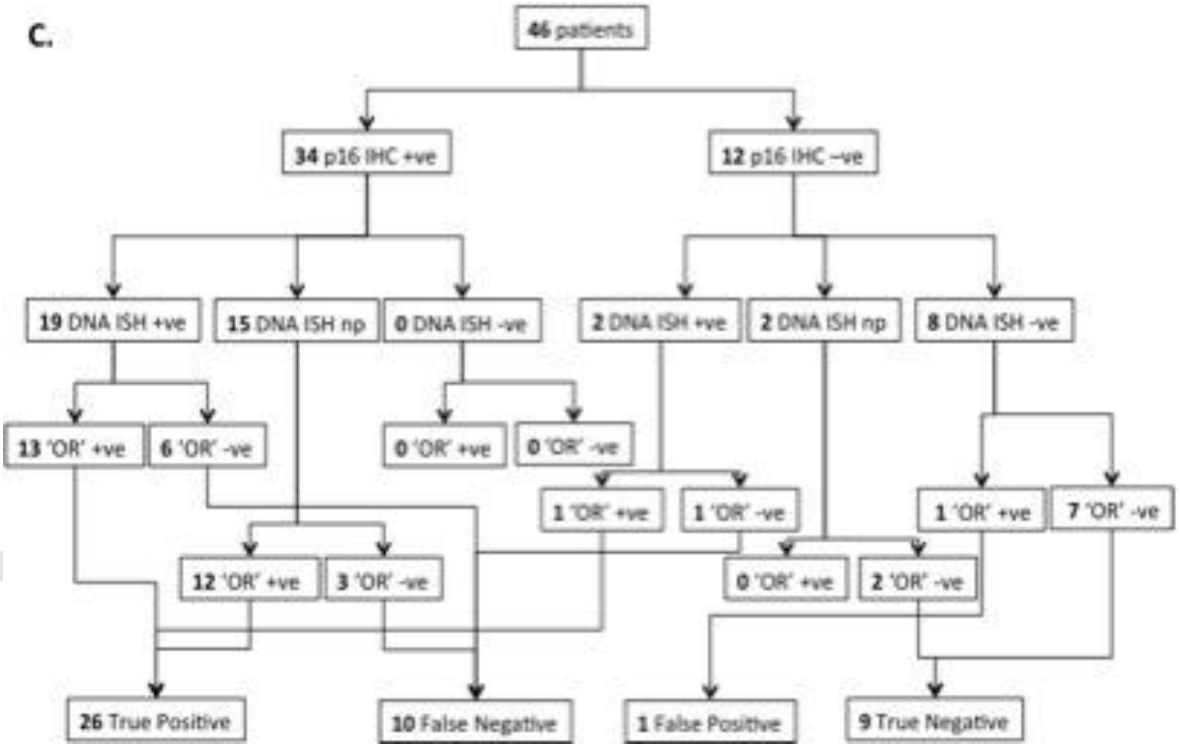

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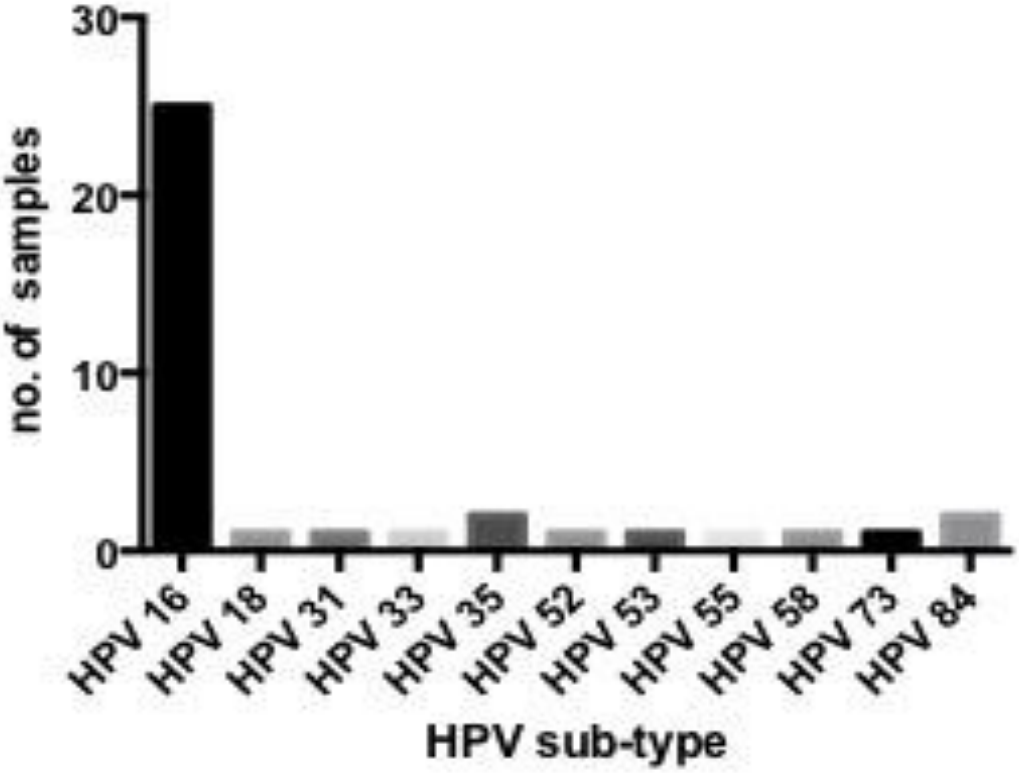

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